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*Short communication*

# Antifeedant activity of citrus limonoids against Colorado potato beetle: comparison of aglycones and glucosides

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## Introduction

The importance of plant allelochemicals in plant-herbivore interactions is well known. Although some of these phytochemicals act as phagostimulants for specific herbivores, the majority of allelochemicals examined appear to function primarily in plant defense, acting as insect antifeedants, growth regulators, and/or toxins (Jermy, 1966; Bernays & Chapman, 1977). As such, these chemicals have great potential for use in pest management, either directly, as natural plant protectants, or as models for the development of synthetic products.

Citrus limonoids, a group of terpenoid allelochemicals found in the plant family Rutaceae have previously been shown to act as insect antifeedants (Klocke & Kubo, 1982; Alford et al., 1987; Mendel et al., 1991) as well as mammalian anticarcinogens (Lam & Hasegawa, 1989; Miller et al., 1989). They are also useful as chemotaxonomic markers (Hasegawa & Miyake, 1996). Thus, citrus limonoids are important functional chemicals in agriculture and medicine.

Limonoid aglycones, which are present in young and growing citrus tissues, are converted to their respective 17$\beta$-D-glucoside derivatives during late stages of fruit growth and maturation (Hasegawa et al., 1991; Fong et al., 1992). Limonoid glucosides, such as limonin 17$\beta$-D-glucopyranoside, are accumulated in mature fruit tissues and seeds as major secondary metabolites (Fong et al., 1989; Hasegawa et al., 1991). Thirty-six limonoid aglycones and 17 limonoid glucosides have been isolated from *Citrus* and its hybrids, yet few have been investigated for their biological activities. None of the limonoid glucosides have previously been examined for antifeedant activity against insects. In this study we compared the antifeedant activity of limonoid aglycones and glucosides against Colorado potato beetle, *Leptinotarsa decemlineata* (Say), larvae.

## Materials and methods

The larvae used in these tests were reared at 24 °C, ca 50% r.h. and a L16:D8 photoperiod on potato foliage (*Solanum tuberosum* L. cv Katahdin) from greenhouse-grown plants and were tested within 24 hrs of molting to fourth stadium. Limonin and isoobacunoic acid were isolated from grapefruit seeds (Herman et al., 1992). Ichangensin and ichangensin glucoside were isolated from Yuzu (*Citrus junos*) seeds (Herman et al., 1989). Limonin glucoside was isolated from citrus molasses (Hasegawa et al., 1996). They were all characterized by NMR analysis. The chemical structures of these compounds are shown in Figure 1.

For the feeding assays, limonin, ichangensin and isoobacunoic acid were dissolved in acetone. The glucosides were dissolved in water with 3% Tween 20 (Sigma, St. Louis, MO) added as a surfactant. Assays were conducted in feeding arenas made of plastic petri dishes (15 × 90 mm) lined with moistened filter paper. Leaf disks (1 cm$^2$) were cut from potato leaves with a No. 8 cork hole borer; then each was coated with either 30 $\mu$l of an acetone solution or 20 $\mu$l of an aqueous solution. Control disks were coated with the solvent

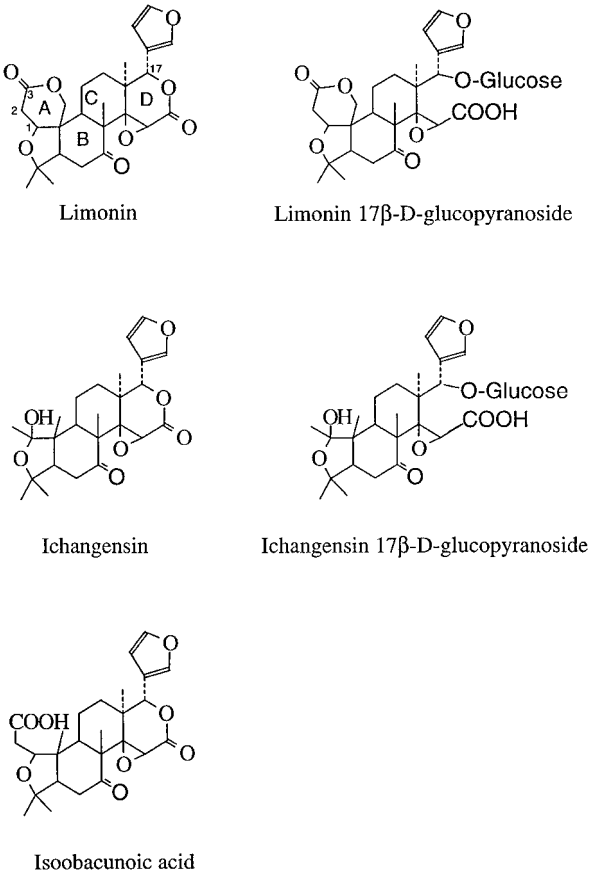


*Figure 1.* Chemical structures of the limonoids.

alone (acetone or 3% aqueous Tween 20 solution). Leaf disks were allowed to air dry, then were placed into the feeding arenas. In each arena, six treated leaf disks were placed equidistant from each other in a circular pattern on top of the filter paper, and one fourth stadium Colorado potato beetle larva was placed in the center.

Feeding responses of Colorado potato beetle to limonin had previously been described (Alford et al., 1987). Therefore, limonin was also included as a reference chemical in each trial to enable direct comparison of its activity to that of each of the test chemicals. Other studies have implicated a chemical receptor-mediated mechanism governing antifeedant responses of Colorado potato beetle to terpenoids (Mullin et al., 1992; Eichenseer & Mullin, 1997). Therefore, we compared activities among solutions of identical molality rather than dosages based on weight to volume ratio. Each test chemical was tested at four dosages: 0, $6.7 \times 10^{-8}$, $2 \times 10^{-7}$, and $6 \times 10^{-7}$ moles/disk. In terms of weight, these dosages were 0, 3, 10, and 30 $\mu$g/disk of ichangensin and isoobacunoic acid; 0, 4.22, 14.05, and 42.15 $\mu$g/disk of ichangensin glucoside; and 0, 4.14, 13.80, and 41.40 $\mu$g/disk of limonin glucoside. The reference compound limonin was tested at 0, $2 \times 10^{-7}$, and $6 \times 10^{-7}$ moles/disk (0, 10, and 30 $\mu$g/disk). After 6–8 hr, the uneaten leaf material was removed from the arenas, dried for 24 h at 40 °C and weighed. The initial leaf disk weight (before feeding) was estimated from the mean dry weight of a separate group of disks cut from the same leaves used in each assay, referred to as 'blank disks'. Consumption was calculated as the mean dry weight of the blank disks minus the dry weight of eaten disks minus the weight of chemical pipetted onto the leaf disks.

Treatment effects on leaf consumption by test insects were determined by Analysis of Variance using SAS PROC GLM (SAS Institute, 1985). Mean separations were determined using Tukey's test (SAS PROC GLM). Log-linear dosage-response effects for each compound were tested with linear contrasts with SAS PROC GLM.

## Results and discussion

Mean consumption of leaf disks treated with the citrus limonoids (Figure 2) indicates that the aglycones were much more active as antifeedants for Colorado potato beetle larvae than the glucosides. Of the aglycones, ichangensin was the most active. Limonin and isoobacunoic acid each caused significant feeding reduction at the highest dosage but were not significantly different from each other. Ichangensin glucoside was active only at the highest dosage, while limonin glucoside did not significantly reduce consumption at any dosage. There was a significant log-linear dosage-response effect of increasing dosage with decreasing consumption for all compounds ($P<0.01$) except limonin glucoside ($P=0.08$).

Our finding that ichangensin is much more active than the other limonoids tested is particularly interesting in light of the taxonomic relationship among the plants producing them. Although limonin occurs widely in the Rutaceae family, ichangensin is present only in *Citrus ichangensis* and its hybrids, including Yuzu (*C. junos*), Sudachi (*C. sudachi*) and Kabosu (*C. sphaerocarpa*) *C. ichangensis* is recognized in Asia where the fruit is used for medicinal purposes. This species belongs to a *Citrus* subgenus, *Papedocitrus*, which has morphological characteristics intermediate to the genus *Citrus* and the *Citrus* subgenus *Papeda*.

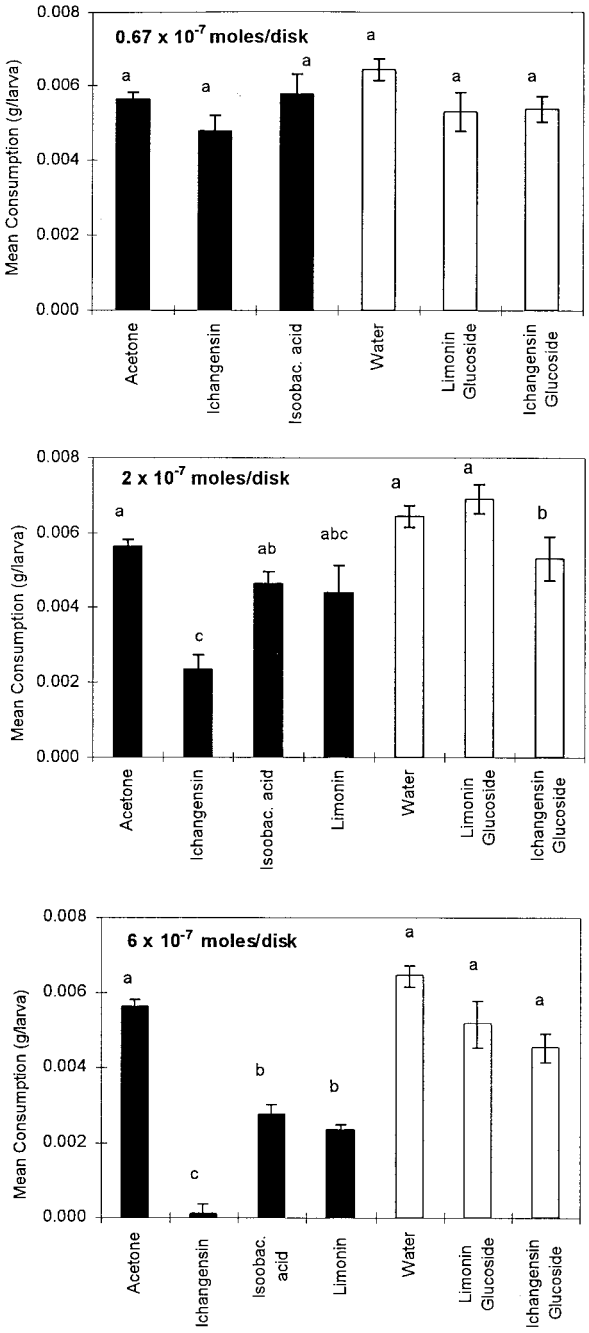


*Figure 2.* Mean amount of potato leaf consumed by Colorado potato beetle fourth stadium larvae offered leaf disks coated with citrus limonoids at three dosages in no-choice assay. Narrow vertical bars indicate ± 1 standard error of the mean. Bars sharing the same letter within each solvent group are not significantly different from one another ($\alpha$=0.05). Note: black bars represent acetone-soluble limonoids (aglycones) and are compared with acetone as a control; open bars represent water-soluble limonoids (glucosides) and are compared with water as a control.

The results of this study lend some insight into the structure-antifeedant activity of the citrus limonoids. Our results show that the A-ring of citrus limonoids is most likely not important for antifeedant activity against Colorado potato beetle. Isoobacunoic acid, which differs from limonin only in substitution of the A-ring with carboxyl group attached to C-2, did not differ from limonin in antifeedant activity. In comparison, ichangensin was highly active, yet it differs from limonin only by a substitution of the A-ring with a hydroxyl group attached to the C-1 position, suggesting that this hydroxyl substitution is directly associated with enhanced activity.

This finding is in contrast to earlier research on Lepidoptera (Klocke & Kubo, 1982; Hassanali et al., 1986), but is consistent with other studies with Colorado potato beetle (Mendel et al., 1991). For instance, it was shown that limonoids with a 7-membered lactone A-ring, such as nomilin and obacunone, are more active antifeedants against fall armyworm (*Spodoptera frugiperda*) and corn earworm (*Helicoverpa zea*) larvae than limonin, a 6-membered A-ring lactone, (Klocke & Kubo, 1982; Hassanali et al., 1986). However, against Colorado potato beetle these 6- and 7-membered A-ring lactone limonoids were found to be of comparable activity (Mendel et al., 1991).

Many naturally occurring glycosidated compounds, which appear to be biologically inactive, accumulate and are stored in mature fruit tissues and seeds. We found here that one D-glucoside attached to the C-17 position of the limonoid molecule with a $\beta$-linkage greatly reduces antifeedant activity. This is also consistent with earlier findings regarding the key role played by the limonoid D-ring in determining antifeedant activity against Colorado potato beetle (Bentley et al., 1988). Glycosidation of the D-ring may alter the conformation of the limonoid molecule sufficiently so that the epoxide and furan groups, essential for Colorado potato beetle antifeedant activity (Bentley et al., 1988) are no longer in an effective position.

Recent studies suggest that beetle chemosensory responses to allelochemicals involve disruption or inhibition of the taste cells normally responding to plant feeding stimulants (van Loon, 1996). Our finding of the drastic change in activity associated with changes in the A-and D-rings suggests a chemical-receptor mediated pathway to antifeedant activity in Colorado potato beetle. Further research is needed to determine the specific mechanisms involved.

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